

# The role of haem in the regulation of rat liver tryptophan metabolism

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1. At saturating concentrations of tryptophan, the activity of tryptophan 2,3-dioxygenase was the same in isolated liver cells and in extracts with added haematin. 2. Intraperitoneal injection of haematin did not increase tryptophan oxidation in livers subsequently perfused *in situ*. 3. Preincubation of liver cells with physiological concentrations of tryptophan caused maximal saturation of tryptophan 2,3-dioxygenase with haem in liver cells. 4. In cell-free extracts tryptophan 2,3-dioxygenase exhibited complex kinetics with haem. 5. The results have important implications for the understanding of the role of haem in tryptophan metabolism.

## INTRODUCTION

In mammals, more than 90% of the total tryptophan is degraded in the liver through the 'kynurenine pathway' (Young *et al.*, 1978). Control of this pathway is important because of the relationship between the plasma concentration of tryptophan and the synthesis of 5-hydroxytryptamine in the brain (Curzon, 1979).

Tryptophan 2,3-dioxygenase (EC 1.13.11.11) is a haem-containing enzyme which catalyses the first reaction unique to tryptophan degradation in the liver. It is a cytosolic enzyme whose activity is regulated chronically by tryptophan (Knox, 1951, 1966; Salter *et al.*, 1984) and by various hormones (Knox & Mehler, 1951; Nakamura *et al.*, 1980; Salter & Pogson, 1985). Under basal conditions the dioxygenase is largely responsible for the control of the pathway; under conditions where enzyme activity is increased, however, control shifts from the dioxygenase to the systems responsible for transport of the amino acid across the plasma membrane (Salter *et al.*, 1985a, 1986a,b).

Rat liver tryptophan dioxygenase exists as the active holo-enzyme and the haem-free apo-enzyme (Feigelson & Greengard, 1961; Knox, 1966; Badawy, 1979; Yamamoto *et al.*, 1981; Stowell & Mørland, 1983; Sadler *et al.*, 1984; Sardana & Drummond, 1986). The ratio of holo- to apo-enzyme is dependent on the availability of haem (Badawy & Evans, 1975; Badawy & Morgan, 1980; Welch & Badawy, 1980; Badawy *et al.*, 1981) and is influenced by agents that affect the conversion of 5-aminolaevulinate into haem, including tryptophan itself (Badawy *et al.*, 1981; Badawy & Morgan, 1982).

Although there have been disagreements about the preferred methodology for determining the degree to which the dioxygenase is saturated with haem in liver homogenates (Badawy *et al.*, 1983; Stowell & Mørland, 1983), it is accepted that a significant proportion of the enzyme in such homogenates is in the unsaturated, or apo-, form. Whether this is the case in whole liver *in vivo*, however, is not clear.

The purpose of the present work was to determine the degree of haem saturation of tryptophan dioxygenase in whole cells and to relate this to the effects of haem as a possible regulator of dioxygenase activity.

A preliminary description of part of this work has been presented (Salter & Pogson, 1986).

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley (University of Manchester) and Wistar (Wellcome Research Laboratories) rats (180–220 g) were used throughout. There was no observable difference in tryptophan metabolism or tryptophan 2,3-dioxygenase between these strains (results not shown).

### Chemicals

L-[ring-2-<sup>14</sup>C]Tryptophan was from CEA, Gif-sur-Yvette, France, through Fluorochem, Glossop, Derbyshire, U.K. [G-<sup>3</sup>H]Tryptophan was from Amersham International, Amersham, Bucks., U.K. Radiolabelled L-isomers were purified by affinity chromatography (Stewart & Doherty, 1973) as described by Salter (1985). 2-Allyl-2-isopropylacetamide was a gift from Roche Products, Welwyn Garden City, Herts., U.K. Haem in the form of haematin hydrochloride, L-tryptophan, dexamethasone phosphate and dimethylformamide were from Sigma. The sources of other reagents were as given previously (Smith & Pogson, 1980; Smith *et al.*, 1980).

### Liver perfusion

All perfusions were started at approx. 13:00 h. Whole livers were perfused *in situ* through the hepatic vein with 200 ml of buffer (Krebs & Henseleit, 1932) at 25 ml/min and 37 °C. This medium was then replaced with 100 ml of the same buffer containing 80 µM-[G-<sup>3</sup>H]tryptophan (sp. radioactivity 3 Ci/mol); this medium was recirculated at the same rate through the liver for 30 min. At 5 min intervals, 0.25 ml samples were taken and mixed with 0.25 ml of a suspension (50 mg/ml in water) of Norit GSX, before centrifugation at 12000 g for 2 min; 0.1 ml of each supernatant was counted for radioactivity of <sup>3</sup>H-labelled non-aromatic products.

### Preparation and incubation of liver cells

Procedures were as described previously (Smith & Pogson, 1980; Smith *et al.*, 1980; Salter *et al.*, 1984),

except that 1.25 mM-CaCl<sub>2</sub> was included in the perfusion medium after addition of collagenase. Glucose (5 mM) was present during cell preparation and incubation. Briefly, flux from tryptophan through tryptophan 2,3-dioxygenase was determined at 37 °C by measuring the release of <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>C-labelled non-aromatic products from [ring-2-<sup>14</sup>C]tryptophan (Smith *et al.*, 1980). Reactions were terminated by the addition of 0.2 ml of 20% (v/v) HClO<sub>4</sub> to incubations.

### Enzyme assay

Tryptophan 2,3-dioxygenase was assayed in whole liver homogenates and liver cell extracts at 37 °C by a modification of the method of Metzler *et al.* (1982). Enzyme activity was measured with 2.5 mM-tryptophan either in the absence (holo-enzyme activity) or in the presence (total enzyme activity) of added haematin (2 µM). Haematin was present from the beginning of the incubation, and enzyme activity was determined from the linear rate of tryptophan metabolism between 40 and 80 min. The haem saturation of tryptophan 2,3-dioxygenase is the percentage of total enzyme activity expressed by the holo-enzyme.

## RESULTS AND DISCUSSION

Table 1 shows the haem saturation of tryptophan 2,3-dioxygenase determined in cell extracts and whole-liver homogenates. These values agree well with values in the literature (Badawy & Evans, 1975; Badawy, 1981; Yamamoto *et al.*, 1981; Stowell & Mørland, 1983; Sadler *et al.*, 1984; Sardana & Drummond, 1986), and demonstrate that, in cell extracts and homogenates, tryptophan 2,3-dioxygenase is far from fully saturated with its cofactor. As expected, addition of further haematin to the incubations did not increase enzyme activity further (results not shown). When cells were incubated with 2.5 mM-tryptophan, the flux through the dioxygenase reaction was  $12.75 \pm 0.95$  nmol/h per mg dry wt.; this rate was not significantly different from that found when enzyme activity was measured in the corresponding cell homogenates supplemented with 2 µM-haematin ( $12.50 \pm 0.60$  nmol/h per mg dry wt.; both values are means  $\pm$  S.E.M. for three separate cell preparations). At lower concentrations of tryptophan, the flux through tryptophan 2,3-dioxygenase in liver cells is limited by substrate supply (Pogson *et al.*, 1984; Salter

*et al.*, 1985b, 1986b). When tryptophan is saturating, however, as in the above experiment, the control coefficient (Salter *et al.*, 1986b) of tryptophan transport for tryptophan degradation is less than 0.02 (results not shown). Under these conditions, therefore, transport of the amino acid will not limit its metabolism to any significant extent.

The degree of haem saturation of tryptophan 2,3-dioxygenase was examined in perfused liver. Fig. 1 shows the metabolism of 80 µM-[G-<sup>3</sup>H]tryptophan by perfused liver *in situ*. Induction of tryptophan 2,3-dioxygenase by pretreatment of the animals with

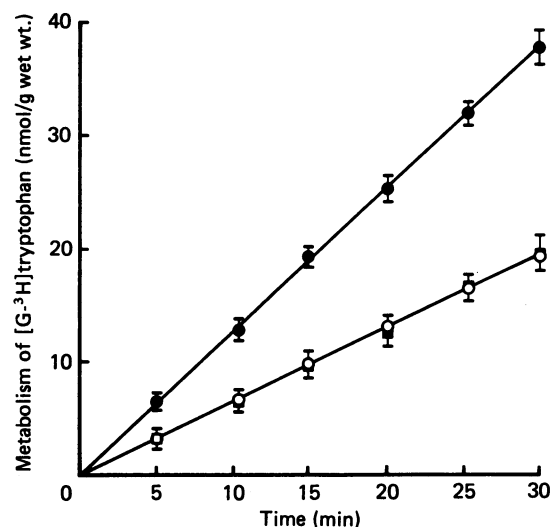


Fig. 1. Effect of haematin and dexamethasone phosphate on the metabolism of [G-<sup>3</sup>H]tryptophan by perfused rat liver

Fed rats were injected intraperitoneally with dexamethasone phosphate (●; 12 mg/kg in 0.9% NaCl, 5 h before perfusion), haematin hydrochloride (■; 6 mg/kg in dimethylformamide, 2 h before perfusion) or vehicle (○; 1 ml/kg in dimethylformamide, 2 h before perfusion). Livers were pre-perfused with 200 ml of buffer as described in the Materials and methods section, with (■) or without (○, ●) 2 µM-haematin hydrochloride. Perfusion was with 80 µM-L-[G-<sup>3</sup>H]tryptophan for 30 min. Samples of the perfusate were taken and treated as described in the Materials and methods section. All results are means  $\pm$  S.E.M. for three animals.

Table 1. Saturation of tryptophan 2,3-dioxygenase with haem in cell extracts and liver homogenates

Liver cells were prepared from fed rats as described in the Materials and methods section. Samples of cell suspensions (2 ml; 10 mg dry wt./ml) were centrifuged (50 g, 2 min); the resulting pellet was extracted by freeze-thawing (three times) with 2 ml of 20 mM-potassium phosphate (pH 7.4)/2.5 mM-tryptophan, with and without 2 µM-haematin hydrochloride. Whole liver tissue was homogenized with 60 vol. (v/w) of the same buffer. Extracts and homogenates were assayed for tryptophan 2,3-dioxygenase activity with and without 2 µM-haematin as described in the Materials and methods section. Results are means  $\pm$  S.E.M. for three animals or separate cell preparations.

|               | Haematin | Activity of tryptophan 2,3-dioxygenase (nmol/h per mg dry wt.) | Saturation with haem (%) |
|---------------|----------|--|--------------------------|
| Cell extracts | —        | $5.60 \pm 0.30$  | $46 \pm 2$               |
|               | +        | $12.95 \pm 0.60$   | $100 \pm 5$              |
| Homogenates   | —        | $5.05 \pm 0.25$  | $48 \pm 2$               |
|               | +        | $10.50 \pm 0.55$   | $100 \pm 5$              |

dexamethasone phosphate (Salter & Pogson, 1985) caused a significant ( $P < 0.001$ ) increase in the rate of metabolism of [ $G\text{-}^3\text{H}$ ]tryptophan; haem saturation of tryptophan 2,3-dioxygenase has been shown to be unaffected by administration of glucocorticoids (Badawy & Evans, 1975). However, pretreatment with haematin, followed by perfusion in the presence of  $2\text{ }\mu\text{M}$ -haematin hydrochloride, yielded rates indistinguishable from those of controls with vehicle alone. The intracellular haem content of liver cells, measured directly by the method of Morrison (1965) (results not shown) or indirectly by the saturation of tryptophan 2,3-dioxygenase (Fig. 2), increased significantly ( $P < 0.001$ ) on incubation of liver cells with  $2\text{ }\mu\text{M}$ -haematin. These data therefore suggest that the concentration of haem remaining in the perfused liver of the control animals was sufficient to maintain total saturation of tryptophan 2,3-dioxygenase.

Badawy has suggested that physiological concentrations of tryptophan increase the conversion of 5-aminolaevulinate into haem by activating 5-aminolaevulinate dehydratase (EC 4.2.1.24) and thereby increase the saturation of tryptophan 2,3-dioxygenase with haem (Badawy *et al.*, 1981; Badawy & Morgan, 1982). Fig. 2 shows the effect of tryptophan on the haem saturation of tryptophan 2,3-dioxygenase in isolated liver cells. It is clear that the enzyme is initially unsaturated with cofactor; however, addition of concentrations of tryptophan that are in the physiological range or below (Madras *et al.*, 1973; Curzon & Knott,

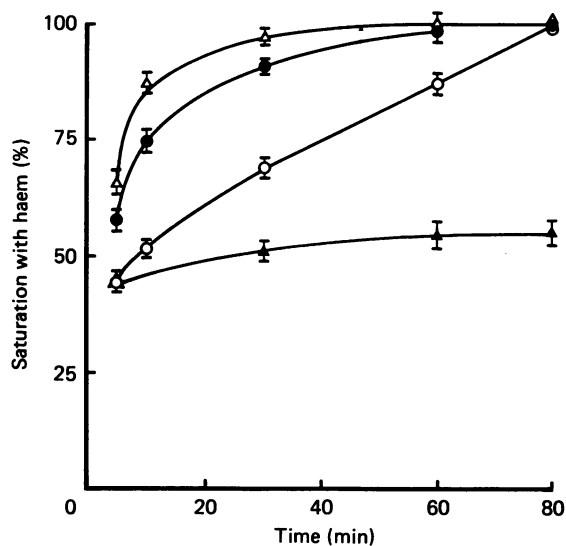


Fig. 2. Effect of tryptophan on the haem saturation of tryptophan 2,3-dioxygenase in isolated liver cells

Liver cells were prepared from fed rats as described in the Materials and methods section. Samples of cells (2 ml; 10 mg dry wt./ml) were preincubated with and without  $2\text{ }\mu\text{M}$ -haematin hydrochloride for 30 min. Tryptophan ( $\blacktriangle$ ,  $0\text{ }\mu\text{M}$ ;  $\circ$ ,  $5\text{ }\mu\text{M}$ ;  $\bullet$ ,  $50\text{ }\mu\text{M}$ ;  $\triangle$ ,  $150\text{ }\mu\text{M}$ ) was then added and incubations were continued for various periods (0, 5, 25, 55 and 75 min) before addition of L-[ring- $2\text{-}^{14}\text{C}$ ]tryptophan (final sp. radioactivity  $3\text{ Ci/mol}$ ). Incubations were terminated 5 min later and were treated as described in the Materials and methods section. Results are means  $\pm$  S.E.M. from three separate cell preparations.

1974; Bloxam *et al.*, 1975; Badawy *et al.*, 1984) resulted in the complete saturation of tryptophan 2,3-dioxygenase with haem.

When liver cells were prepared in the presence of  $5\text{ }\mu\text{M}$ -tryptophan, and were then incubated with  $5\text{ }\mu\text{M}$ -tryptophan for 30 min, the flux through tryptophan 2,3-dioxygenase over this period was similar both with and without exogenous  $2\text{ }\mu\text{M}$ -haematin, indicating that the enzyme was fully saturated with its cofactor in the freshly prepared cells (cf. Fig. 2). It is therefore likely that the partial haem saturation of tryptophan 2,3-dioxygenase shown in Fig. 2 was due to a decrease in intracellular haem as a result of preparation of the liver cells in the absence of tryptophan. *In vivo*, the presence of normal plasma concentrations of free tryptophan ( $5\text{--}20\text{ }\mu\text{M}$ ) (Madras *et al.*, 1973; Curzon & Knott, 1974; Bloxam *et al.*, 1975; Badawy *et al.*, 1984) will therefore probably be sufficient to maintain tryptophan 2,3-dioxygenase in a fully haem-saturated state. The effect of preincubation of tryptophan with liver cells on the haem-saturation of tryptophan 2,3-dioxygenase, assayed in cell-free extracts, is shown in Table 2. Tryptophan was clearly much less effective as a promoter of haem saturation than in the experiments described above (cf. Fig. 2). This is attributable to the dilution of the intracellular haem pool during extraction. However, incubation of liver cells with  $100\text{ }\mu\text{M}$ -tryptophan increased haem sufficiently to saturate the enzyme totally, even after dilution on homogenization (cf. Badawy *et al.*, 1981). It therefore appears that assay of the extracted enzyme with and without haematin is inappropriate as a way of determining the saturation of tryptophan 2,3-dioxygenase with its cofactor *in vivo*. Incubation of liver cells with  $100\text{ }\mu\text{M}$ -tryptophan (Table 2) not only promoted total haem saturation of tryptophan 2,3-dioxygenase but also increased its total activity. This is consistent with claims that *in vivo* tryptophan increases tryptophan 2,3-dioxygenase protein by decreasing its rate of degradation (Schimke *et al.*, 1965).

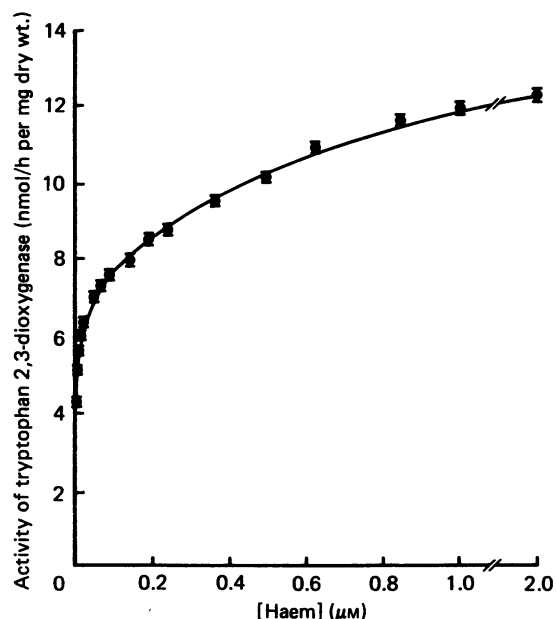
Fig. 3 shows the relationship between haem concentration and dioxygenase activity in cell-free extracts. The enzyme exhibited complex kinetics with haem; this is consistent with the hypothesis that tryptophan 2,3-dioxygenase possesses at least two distinct haem-binding sites. Many groups have reported haem saturations of approx. 50% in whole liver homogenates (Yamamoto *et al.*, 1981, 1982; Badawy & Morgan, 1982, and references therein; Stowell & Mørland, 1983; Sadler *et al.*, 1984; Sardana & Drummond, 1986). It is probable that, on homogenization and consequent dilution, haem will dissociate from the low-affinity site(s); the high-affinity site(s) will, however, retain most of the haem until very low haem concentrations are reached. Haem from the high-affinity site(s) will only dissociate extensively under conditions of severe haem depletion, e.g. after treatment with a porphyrinogen such as 2-allyl-2-isopropylacetamide and subsequent dilution of the free haem pool by homogenization (Badawy & Morgan, 1980).

It has been reported that tryptophan 2,3-dioxygenase in some species such as the guinea pig exists exclusively as holo-enzyme when assayed in liver homogenates (Hvitfelt & Santti, 1972; Badawy & Evans, 1974). One might speculate that the guinea-pig enzyme possesses only high-affinity haem-binding sites and that haem concentrations present in liver homogenates are therefore sufficient to maintain saturation of the enzyme.

**Table 2. Effect of incubation of liver cells with tryptophan on the haem saturation of tryptophan 2,3-dioxygenase in cell extracts**

Liver cells were prepared from fed rats as described in the Materials and methods section. Samples (2 ml; 10 mg dry wt./ml) were incubated with and without tryptophan for 90 min before centrifugation at 50 g for 2 min. Pellets were extracted and assayed for dioxygenase activity, with and without haematin, as described in Table 1. Results are means  $\pm$  S.E.M. for three liver cell preparations.

| Tryptophan ( $\mu$ M) | Haematin (2 $\mu$ M) | Activity of tryptophan 2,3-dioxygenase (nmol/h per mg dry wt.) | Saturation with haem (%) |
|-----------------------|----------------------|--|--------------------------|
| 0                     | —                    | 4.30 $\pm$ 0.25  | 35 $\pm$ 2               |
|                       | +                    | 12.30 $\pm$ 0.75   | 100 $\pm$ 6              |
| 5                     | —                    | 5.65 $\pm$ 0.25  | 46 $\pm$ 2               |
|                       | +                    | 12.35 $\pm$ 0.65   | 100 $\pm$ 5              |
| 25                    | —                    | 10.20 $\pm$ 0.45   | 75 $\pm$ 3               |
|                       | +                    | 13.65 $\pm$ 0.40   | 100 $\pm$ 3              |
| 100                   | —                    | 21.60 $\pm$ 1.50   | 104 $\pm$ 7              |
|                       | +                    | 20.80 $\pm$ 1.35   | 100 $\pm$ 6              |

**Fig. 3. Effect of haematin hydrochloride on tryptophan 2,3-dioxygenase activity in liver cell extracts**

Liver cells were prepared from fed rats as described in the Materials and methods section, and 2 ml samples (10 mg dry wt./ml) were incubated in the presence of 150  $\mu$ M-2-allyl-2-isopropylacetamide for 60 min and then centrifuged (50 g, 2 min), washed in 10 ml of the above medium in the absence of 2-allyl-2-isopropylacetamide, centrifuged (50 g, 2 min) and the supernatant discarded. The cell pellet was extracted and assayed for tryptophan 2,3-dioxygenase activity as described in Table 1. 2-Allyl-2-isopropylacetamide was added to cell incubations to decrease the haem concentration and thus cause more apo-tryptophan 2,3-dioxygenase to be present for titration with exogenous haematin (Badawy & Morgan, 1980). Results are means  $\pm$  S.D. from one liver cell preparation.

### General discussion

The major conclusion to be drawn from these observations is that tryptophan 2,3-dioxygenase is fully saturated with its cofactor *in vivo*. It appears that even large decreases in the concentration of intracellular free

haem may be ineffective in depleting flux through the kynurenine pathway; increases in haem will certainly be ineffective. Fluctuation in the liver tryptophan concentration (and thus haem concentration) is therefore unlikely to precipitate changes in the rate of tryptophan metabolism through changes in the saturation of tryptophan 2,3-dioxygenase with haem. Measurements of the saturation of tryptophan 2,3-dioxygenase with haem in liver homogenates clearly do not reflect the haem status of tryptophan 2,3-dioxygenase *in vivo*. However, as a qualitative indication of the free haem available to tryptophan 2,3-dioxygenase and 5-aminolaevulinate synthase (Badawy & Morgan, 1982), such measurements may have some validity.

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